



Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/microbres



Diversity and antifungal activity of the endophytic fungi associated with the native medicinal cactus *Opuntia humifusa* (Cactaceae) from the United States

Alice F. Silva-Hughes^a, David E. Wedge^{b,*}, Charles L. Cantrell^b, Camila R. Carvalho^a, Zhiqiang Pan^b, Rita M. Moraes^{c,d}, Victor L. Madoxx^e, Luiz H. Rosa^{a,**}

^a Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^b Natural Products Utilization Research Unit, Agricultural Research Service, United States Department of Agriculture, University, MS, USA

^c National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS, USA

^d Center for Water and Wetland Resources, University of Mississippi, Abbeville, MS, USA

^e Geosystems Research Institute, Mississippi State University, MS, USA

ARTICLE INFO

Article history:

Received 19 February 2015

Received in revised form 8 March 2015

Accepted 11 March 2015

Available online xxx

Keywords:
Cacti
Diversity
Endophytes
Fungi
Pesticides

ABSTRACT

The endophytic fungal community associated with the native cactus *Opuntia humifusa* in the United States was investigated and its potential for providing antifungal compounds. A hundred-eight endophytic fungal isolates were obtained and identified by molecular methods into 17 different taxa of the genera *Alternaria*, *Aureobasidium*, *Biscogniauxia*, *Cladosporium*, *Cryptococcus*, *Curvularia*, *Diaporthe*, *Epicoccum*, *Paraconiothyrium*, *Pestalotiopsis* and *Phoma*. The most frequent species associated with *O. humifusa* were *Alternaria* sp. 3, *Aureobasidium pullulans* and *Diaporthe* sp. The fungal community of *O. humifusa* had a high richness and diversity; additionally, the species richness obtained indicates that the sample effort was enough to recover the diversity pattern obtained. Six extracts of endophytes showed antifungal properties and ¹H NMR analyses of the extracts of *Alternaria* sp. 5 Ohu 8B2, *Alternaria* sp. 3 Ohu 30A, *Cladosporium funiculosum* Ohu 17C1 and *Paraconiothyrium* sp. Ohu 17A indicated the presence of functional groups associated with unsaturated fatty-acid olefinic protons and fatty acid methylene and methyl protons. GC-FID analysis of these extracts confirmed the presence of a mixture of different fatty acids. The ¹H NMR analyses of *Biscogniauxia mediterranea* Ohu 19B extracts showed the presence of aromatic compounds. From the extract of *B. mediterranea* we isolated the compound 5-methylmellein that displayed moderate antifungal activity against the phytopathogenic fungi *Phomopsis obscurans*. Our results suggest that native medicinal cacti of the United States can live symbiotically with rich and diverse endophytic communities and may be a source of bioactive molecules, including those able to inhibit or control plant disease pathogens.

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Introduction

Endophytic fungi are those living inside plant tissues or organs, without causing them any harmful symptoms (Petri 1991), including giving greater host plant resistance to biotic and/or abiotic stresses. The fungal species that have been reported to be endophytic of different plant species include mainly Ascomycota, Basidiomycota and Zygomycota. Different studies reported that

plants colonized by endophytic fungi represent an important repository of the fungal diversity and new species (e.g., Carvalho et al. 2012; Tao et al. 2013).

The endophytic fungi seem to produce bioactive compounds, originally isolated from their host plants, as well as bioactive metabolites that are clearly different from those of plants and feature unique structural characteristics, which may have potential use in agriculture and medicine (Stierle et al. 1995; Puri et al. 2005; Wang et al. 2013). According to Kumar and Kaushik (2012), different biofungicide metabolites, such as alkaloids, terpenoids, steroids, isocoumarins and chromones, phenolics and volatiles isolated and characterized from endophytic fungi display antifungal activity against plant pathogenic fungi. The endophytic fungi may provide agrochemical agents, which may be used as

* Corresponding author. Tel.: +1 601 232 1134; fax: +1 601 232 1035.

** Corresponding author. Tel.: +55 31 3409 2749; fax: +55 31 3409 2730.

E-mail addresses: dwedge@olemiss.edu (D.E. Wedge), [\(L.H. Rosa\).](mailto:lhrosa@icb.ufmg.br)

an alternative to replace synthetic pesticides, considering the increasing incidence of chemical resistance in fungal pathogens and potential environmental and mammalian toxicities (Kumar and Kaushik 2012; Wang et al. 2013).

The genus *Opuntia* (Cactaceae) includes about 2300 species and is native to the Americas (Powell and Weedon 2004; Majure 2010). *Opuntia* species have been used widely as ornamental plants, food-stuffs, forage crop and as medicinal plants in arid areas of the world, such as parts of Brazil, Mexico, western Asia and northern and southern Africa (Inglese et al. 2002; Nefzaoui and Salem 2002; Majure and Ervin 2008). *Opuntia humifusa* (Raf.) Raf., a cold tolerant cactus, is commonly known as Eastern Prickly Pear and/or devil's tongue and probably originated in the southwestern United States and adjacent northern Mexico and then dispersed eastward into the southeastern US (Nobel 1996; Majure 2010). Devil's tongue is rich in polyphenols and flavonoids, minerals and is a good source of dietary fiber (Hahm et al. 2011). *O. humifusa* shows different biological activities, including promoting anti-atherosclerosis (Kwon and Song 2005), anticancer (Yoon et al. 2009a,b), antibacterial (Lee et al. 2004), antioxidant (Lee et al. 2005a; Park et al. 2005), anti-inflammatory (Lee et al. 2005b), hypoglycemic and hypolipidemic activity (Kwon and Song 2005) and an alternative treatment of post-menopausal osteoporosis (Park et al. 2011). After all information below, the aim of the present work was to examine the diversity of the endophytic fungal community associated with *O. humifusa* and investigate antifungal potential against phytopathogenic fungal species.

Methods and materials

Sample collection and isolation of fungal endophytes

Adult specimens of *O. humifusa* (Raf.) Raf (Cactaceae) (Fig. 1) were randomly collected from two area: site 1 (N36.86140 W90.95036) and site 2 (N36.91370 W89.53330) in July 2012 at Missouri state, USA. Three stems were collected from 18 adult specimens of *O. humifusa* per study area (a total of 36 individuals). The stems were placed in disinfected individual plastic bags and

stored for less than 24 h at 10 °C prior to the isolation of endophytic fungi. Fifteen fragments of stems were plated, totaling 15 fragments per plant and 540 fragments in all. Before surface disinfection, the stems were washed and cut into 5-mm-long fragments using a flame-sterilized scalpel in a laminar flow hood to avoid air contamination of the tissues and all samples were processed separately. The fragments were surface disinfected by immersion 70% ethanol (1 min), 2% sodium hypochlorite (3 min) and washed with sterile distilled water (2 min). The fragments were placed on Petri plates containing potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, Missouri) supplemented with chloramphenicol (200 µg/mL) (Sigma/USA). The plates were incubated for up to 60 days at 25 °C and the hyphal tip of each fungus growing out from the plant tissue was excised and transferred to a new PDA plate. After incubation at 25 °C for 10 days, culture purity was assessed using colony morphology. To test the effectiveness of the surface disinfection, 100 µL of the last water rinse was plated on PDA and incubated at 25 °C. Long-term samples of the filamentous fungal colonies were stored in cryotubes with 20% sterile glycerol at -80 °C.

Endophytic fungal identification

Filamentous fungal isolates were grouped into different morphospecies according to their characteristics of the culture: colony color and texture, border type, and radial growth rate on PDA agar (Fröhlich et al. 2000). To produce fungal mycelia, the isolates were grown in potato dextrose broth (PDB, Sigma-Aldrich, St. Louis, Missouri) incubated in a water bath (100 rpm) at 25 ± 0.5 °C for 5 days. The mycelium was washed twice in sterile tap water, centrifuged for 10 min at 4000 × g, and then freeze-dried. The freeze dried mycelia were stored in eppendorf tubes at -20 °C until DNA extraction.

All endophytic fungi were identified by molecular methods. DNA from endophytic fungi was extracted with a DNeasy Plant Mini kit (Qiagen Inc., Valencia, California), but modified in the cell lysis by Fredlund et al. (2008) and used as a template in PCR amplifications. Isolates with identical morphological characteristics were grouped together and subjected to PCR fingerprinting using the

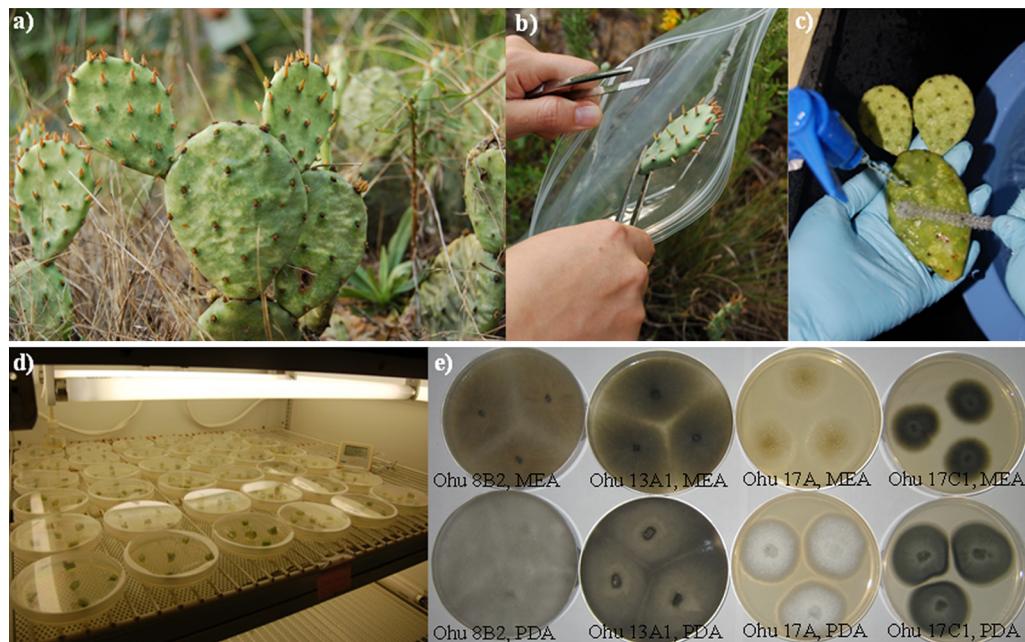


Fig. 1. Plant material and isolation process of the endophytic fungi. Adult specimens of *Opuntia humifusa* (a), stem collected from adult specimens of *O. humifusa* (b), stems washed with sterile distilled water (c), fragments placed on Petri plates containing potato dextrose agar (PDA) and incubated at 25 °C (d), and bioactive filamentous fungal isolates recovered from *O. humifusa* on PDA and Malt Extract Agar (MEA) (e).

Table 1Molecular identification of endophytic fungi associated with *Opuntia humifusa*.

Ohu ^a	No. of isolates	Top BLAST search results [GenBank accession number]	Query coverage (%)	Identity (%)	No. of bp sequenced and analyzed	Proposed species or taxonomic group [GenBank accession number]
5B1 ^e	29	<i>Aureobasidium pullulans</i> [FN824492]	100	100	526	<i>Aureobasidium pullulans</i> [KF850387] ^b
13A1 ^e	18	<i>Alternaria alternata</i> [KF438091]	100	100	519	<i>Alternaria</i> sp. 3 [KJ471579 ^b ; KR023652 ^c]
8A ^e	14	<i>Diaporthe phaseolorum</i> [EU301113]	100	99	519	<i>Diaporthe</i> sp. [KF850394 ^b ; KR023657 ^c ; KR023649 ^d]
26A ^e	10	<i>Alternaria alternata</i> [JQ070079]	100	100	540	<i>Alternaria</i> sp. 4 [KF850386 ^b ; KR023651 ^c]
7A ^e	9	<i>Alternaria alternata</i> [KC959211]	100	100	516	<i>Alternaria</i> sp. 2 [KF850385 ^b]
2B ^e	8	<i>Cladosporium cladosporioides</i> [JQ768318]	100	100	549	<i>Cladosporium</i> sp. [KF850389] ^b
2A ^e	5	<i>Cochliobolus</i> sp. [JX960581]	100	100	570	<i>Curvularia protuberata</i> [KF850392] ^b
17C1 ^e	4	<i>Cladosporium cladosporioides</i> [JX981454]	100	100	523	<i>Cladosporium funiculosum</i> [KF850391 ^b ; KR023656 ^c]
17A ^e	2	<i>Paraconiothyrium sporulosum</i> [EU821483]	100	99	570	<i>Paraconiothyrium</i> sp. [KF850396 ^b]
18B1 ^e	2	<i>Pestalotiopsis clavigpora</i> [KC537808]	100	100	461	<i>Pestalotiopsis</i> sp. [KF850397 ^b]
4B ^e	1	<i>Alternaria alternata</i> [KC692221]	100	100	550	<i>Alternaria</i> sp. 1 [KF850384 ^b ; KR023650 ^c]
8B2 ^e	1	<i>Alternaria alternata</i> [KF155521]	100	100	504	<i>Alternaria</i> sp. 5 [KJ471580 ^b ; KR023653 ^c]
19B ^f	1	<i>Biscogniauxia mediterranea</i> [EF026134]	100	100	530	<i>Biscogniauxia mediterranea</i> [KF850388 ^b ; KR023654 ^c , KR023648 ^d]
2C1 ^e	1	<i>Cladosporium bruhnei</i> [KC461496]	99	99	551	<i>Cladosporium</i> cf. <i>asperulatum</i> [KF850390 ^b ; KR023655 ^c]
7B	1	<i>Cryptococcus flavescens</i> [KC679297]	100	100	409	<i>Cryptococcus flavescens</i> [KF850393] ^b
23A ^f	1	<i>Epicoccum nigrum</i> [KC164754]	100	100	435	<i>Epicoccum nigrum</i> [KF850395] ^b
11B ^e	1	<i>Phoma novae-verbascicola</i> [KC411473]	100	100	515	<i>Phoma</i> sp. [KF850398 ^b]

^a Ohu = culture code of endophytic fungi.^b ITS sequences deposited.^c TEF1 sequences deposited.^d β -Tubulin sequences deposited.^e Taxonomic position suggested by the phylogenetic analyses.^f Taxonomic position confirmed by the phylogenetic analyses.

microsatellite-primed PCR technique (GTG)₅ by Lieckfeldt et al. (1993). Based on the electrophoretic profile of PCR-amplified products with primer (GTG)₅, an isolated among those who showed the same pattern of bands was selected for sequencing the internal transcribed region of the nuclear ribosomal RNA gene (ITS1-5.8S-ITS2), translation elongation factor 1 α gene (TEF1) and/or β -tubulin gene (B-TUB). The ITS1-5.8S-ITS2 genomic region was amplified from genomic DNA using the forward primer ITS1 and the reverse primer ITS4 (White et al. 1990). The primers EF1-728F and EF1-986R (Carbone and Kohn 1999) were used to amplify part TEF1 and the primers T1 (O'Donnell and Cigelnik 1997) and Bt-2b (Glass and Donaldson 1995) to amplify part of the B-TUB. For ITS region and B-TUB gene the amplification reactions and PCR conditions were performed by Godinho et al. (2013) and for TEF1 gene were by Carbone and Kohn (1999). After amplifications, an aliquot was analyzed by electrophoresis on a 1% TAE agarose gel, visualized under UV light and PCR products were compared to the molecular size standard 1 kb plus DNA ladder (Invitrogen, CA). Successfully amplified PCR products were extracted using MinElute PCR Purification Kit (Invitrogen, CA) and sequenced on an automated DNA Sequencer (model ABI 3730XL; Applied Biosystems, Foster City, CA). Consensus sequence data of the endophytic fungi were submitted to the GenBank database (see accession numbers in Table 1).

Phylogenetic analysis

The sequences of filamentous fungi were aligned using method MUSCLE (Edgar 2004) with manual adjustments for visual improvement where necessary, in program MEGA version 5.0 (Tamura et al. 2011). The alignments for each studied genus were prepared including sequences of all relevant ex-type strains, from culture collection and/or published indexed journals sequences obtained from GenBank to ensure an accurate identification. Alignment gaps were coded as fifth base in the analysis. For each set of ITS, TEF1 and β -tubulin sequences and combined analysis for undetermined endophytic taxa. Phylogenetic trees were constructed using maximum parsimony method (MP) using programs contained in PAUP

Phylogenetic Software version 4.0b10 (Swofford 2002). Parsimony trees were reconstructed under the constraints of each hypothesis by heuristic search using tree bisection-reconnection (TBR) branch swapping on starting trees generated by random sequence addition (100 reps). The unconstrained topologies of the equally parsimonious trees were compared using the Kishino-Hasegawa test of PAUP. The best topology was selected as the most parsimonious tree topology. Parsimony bootstrap with 5000 replicates in PAUP was applied to the tree to evaluate the stability. Additionally, combined analysis for the inconclusive fungal taxa phylogenetic trees were also constructed using Bayesian inference (BA), in order to check the occurrence of possible failures related to the method of phylogenetic inference (Felsenstein 1985; Swofford et al. 2001; Bergsten 2005). We chose the best evolutionary models for each data partition using the software MrModelTest v. 2.3 (Nylander et al., 2004) based on the values of the Akaike information criterion (AIC). Metropolis coupled/Markov chain/Monte Carlo (MCMC) analysis was conducted on the combined ITS1-5.8S-ITS2, TEF1 and B-TUB data set using MrBayes 3.2 (Ronquist et al. 2012). MCMC searches were run for 2,000,000 analyzes of four chains was started in parallel from a random tree topology, and sampled every 100 generations, resulting in an overall sampling of 20,000 trees. Values of the average standard deviation of split frequencies (ASDSF) came below 0.01 and potential scale reduction factor (PSRF) among 0.98–1.02 were considered as indicators of stationarity. Bayesian posterior probabilities (PP) was computed from a majority rule consensus tree after 25% burn-in. Clades with PP > 0.95 were considered supported.

Ecological analyses

In order to evaluate the species composition and diversity parameters for all hosts, we used the inverse of the Simpson index ($E_{1/D}$) (Magurran 2004) to estimate the evenness and rank-abundance dominance (RDA) (Whittaker 1972) to explore the dominance distribution of the endophytic community. The best model was selected by Schwartz's Bayesian information criteria

(BIC). The sampling sufficiency was evaluated using Species accumulation curves generated by 1000 randomizations of *O. humifusa* plants (Kindt et al. 2006). Two incidence-based estimators of the total number of species (Jackknife 1 and Bootstrap) were calculated (Magurran 2004). To analyze the relative contribution of each host plant to the endophytic gamma diversity in *O. humifusa*, we used the contribution partition analysis proposed by Liu et al. (2007). According to the partition of species diversity, where the regional (gamma) diversity is the sum of the host (alpha) and inter-host (beta) diversity, an index of relative contribution of each term could be estimated. A value of $D_{ST} > 0.5$ means that most diversity is distributed among units (Liu et al. 2007). Furthermore, the significant difference between two sites based on Bray–Curtis distance measurement was statistically test with nonparametric procedure ANOSIM (Clarke 1993) and Whittaker's coefficient of beta diversity [Whittaker, 1960; hereafter $\beta(W)$]. $\beta(W)$ is a direct measure of the overlap among sites. All the diversity statistical analyses were performed as implemented in R package “vegan” (Oksanen et al. 2012). The significant difference between two sites based on Bray–Curtis distance measurement was statistically test with nonparametric procedure ANOSIM (Clarke 1993).

Extract preparation from fungus and plant

The crude extract productions follow the protocols established by Rosa et al. (2013). Briefly, a five mm diameter plug of fungus was placed on 20 mL of PDA (Difco, Detroit, MI) medium at the center of 200 Petri dishes (90 mm diameter) and cultured for 15 days at $25 \pm 2^\circ\text{C}$ (time enough for endophytic fungi produce secondary metabolites). These fungal cultures were lyophilized for 72 h, cut in small pieces and transferred to 50 mL glass centrifuge tubes followed by the addition of 50 mL of dichloromethane (DCM, Fisher, USA). After 72 h at room temperature, the organic phase was filtered and the solvent removed under rotary evaporation at 40°C . An aliquot of dried extract was dissolved in water/methanol (1:1) in order to prepare a 20 mg/mL stock solution, which was stored at -20°C until needed. Sterile PDA medium was extracted using the same procedure. The sterile PDA extract was used as the control in the screening procedure. Five plates which were not inoculated with the fungus were subjected to the same protocol to serve as controls of the culture medium. Stems of the same *O. humifusa* used for isolation of all endophytic fungi and the sterile PDA (five plates not inoculated) were subjected to the same protocol.

Antifungal bioautography assay

Bioautography procedures were described in our previous studies (Wedge et al. 2009). The DCM extract of endophytic fungi and plants were applied at 160 $\mu\text{g}/\text{spot}$ in chloroform onto a silica plate. Technical fungicide grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service Inc., West Chester, PA, USA) were used as positive controls at 2 mM in 2 μL of 95% ethanol and the sterile PDA extract was used as the negative control.

Microdilution broth assay

A reference method [M27-A from the National Committee for Clinical Laboratory Standards (NCCLS)] for broth-dilution antifungal susceptibility testing of yeast was adapted for evaluation of antifungal compounds against sporulating filamentous fungi (Wedge and Kuhajek 1998). The 96-well microtiter assay was used to determine the sensitivity of *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Fusarium oxysporum*, *Botrytis cinerea*, *Phomopsis obscurans*, and *P. viticola* to the various antifungal agents in comparison with several commercial fungicides. The

fungicides azoxystrobin and captan (0.3, 3 and 30 μM) were used as standards in these assays. Each fungal species was challenged in a dose–response format so that the final test compound concentrations of 75, 150 and 300 μM were achieved (in duplicate) in the different columns of the 96-well plate. Each compound was evaluated in duplicate, and the experiment was performed three times. Mean absorbance (at 620 nm) and standard errors were used to evaluate fungal growth after 48 and 72 h except for *P. obscurans* and *P. viticola* (120 and 144 h).

Instrumentation

^1H and ^{13}C NMR spectra were recorded in CDCl_3 on a Bruker Avance 400 MHz spectrometer (Billerica, MA). All ^{13}C multiplicities were deduced from 90° and 135° DEPT experiments. Gas chromatography–mass spectrometry (GC–MS) analysis was performed on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. High-resolution mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC) (Peabody, MA). Column chromatography was performed using a Biotage, Inc., Horizon Pump (Charlottesville, VA) equipped with a Horizon Flash Collector and fixed wavelength (254 and 280 nm) detector.

GC–MS analysis

Reaction products were analyzed by GC–MS using a DB-5 column ($30\text{ m} \times 0.25\text{ mm}$ fused silica capillary column, film thickness of $0.25\text{ }\mu\text{m}$) operated using the following conditions: injector temperature, 240°C ; column temperature, $60\text{--}240^\circ\text{C}$ at $3^\circ\text{C}/\text{min}$ then held at 240°C for 5 min; carrier gas, He; and injection volume, 1 μL (splitless).

High-resolution LC–MS analysis

The isolated compound was prepared in methylene chloride and injected directly into a $0.4\text{ mL}/\text{min}$ stream of a 20% $\text{H}_2\text{O}/80\%$ MeOH solution containing 1 $\mu\text{g}/\text{mL}$ L-tryptophan. Mass drift compensations were performed relative to L-tryptophan [$\text{M}+\text{H}]^+$ and/or $[2\text{M}+\text{H}]^+$ ions.

Fatty acid reference standards and methyl ester synthesis

Fatty acid abbreviations used throughout the manuscript are identified in parentheses. Individual saturated fatty acids hexanoic acid ($\text{C}_{6:0}$), heptanoic acid ($\text{C}_{7:0}$), octanoic acid ($\text{C}_{8:0}$), nonanoic acid ($\text{C}_{9:0}$), and tridecanoic acid ($\text{C}_{13:0}$), and individual unsaturated fatty acids undecanoic acid ($\text{C}_{11:1}$), 11-dodecanoic acid ($\text{C}_{12:1}$), 12-tridecanoic acid ($\text{C}_{13:1}$), myristoleic acid ($\text{C}_{14:1}$), and palmitoleic acid ($\text{C}_{16:1}$) were all purchased from Nu-Chek Prep, Inc. (Elysian, MN). Decanoic acid ($\text{C}_{10:0}$), undecanoic acid ($\text{C}_{11:0}$), dodecanoic acid ($\text{C}_{12:0}$), tetradecanoic acid ($\text{C}_{14:0}$), hexadecanoic acid ($\text{C}_{16:0}$), octadecanoic acid ($\text{C}_{18:0}$), oleic acid ($\text{C}_{18:1}$), and linoleic acid ($\text{C}_{18:2}$) were all purchased from Sigma–Aldrich (St. Louis, MO). Fatty acid methyl esters used in the analyses of extracts and fractions were either synthesized from the corresponding free fatty acids (see diazomethane reaction below) or analyzed as a premixed reference standard from Supelco (St. Louis, MO), part number 18919.

Free fatty acids were converted to their corresponding methyl esters by reacting them with diazomethane in ether. An Aldrich (St. Louis, MO) Mini Diazald Apparatus was used for the production of diazomethane in ether. Briefly, 2.5 g of KOH was dissolved in 4 mL of deionized water and placed in the reaction vessel followed by the addition of 5 mL of ethanol. A separatory funnel containing 2.5 g of diazald dissolved in 22.5 mL of ether was placed above the reaction vessel. The reaction vessel was warmed to 65°C using a

water bath followed by the drop wise addition of the diazald solution over a period of 50 min. The receiving flask and condenser cold finger were cooled using a dry-ice/acetone bath. The co-distilled diazomethane (CH_2N_2) in ether solution was stored in sealed vials at -20°C until needed. One mg of free fatty acids or fractions in 500 μL of diethyl ether was treated at room temperature overnight with a solution of CH_2N_2 in diethyl ether (500 μL). Solvent and residual CH_2N_2 were removed under a stream of N_2 and the sample was then re-dissolved in diethyl ether for GC analysis.

Gas chromatography – flame ionization detection (GC-FID) analysis

GC-FID analysis was performed on a Varian CP-3800 GC. The GC was equipped with a DB-23 (Agilent Technologies) column (60 m \times 0.25 mm capillary column, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 270°C ; column temperature, 130°C held for 1 min followed by 130 – 170°C at $6.5^\circ\text{C}/\text{min}$ followed by 170 – 215°C at $2.8^\circ\text{C}/\text{min}$ and held for 12 min followed by 215 – 230°C at $40^\circ\text{C}/\text{min}$ and held for 3 min; injection volume, 11(split 20:1); 3 mL/min constant flow; FID temperature of 300°C . Fatty acid methyl esters were identified by injection of commercially available standards and comparison of retention times with that of unknowns. The fatty acids were quantified by performing area percentage calculations based on the total combined FID area. For example, the area for each reported peak was divided by the total integrated area from the FID chromatogram from all reported peaks and multiplied by 100 to arrive at a percentage. The percentage is a percentage by weight relative to all other constituents integrated in the FID chromatogram.

Isolation and identification of antifungal compounds

Initially, 1.0 g of the *B. mediterranea* Ohu 19B extract was adsorbed to silica gel and applied to a silica gel chromatography column (40–63 μm , 40 \times 150 mm, 60 Å). For the *B. mediterranea* Ohu19B extract the elution of the column was performed using increasing polarity mixtures of hexane/EtOAc in a series of three linear steps as follows: (step 1) 100:0 to 98:2 using 396 mL, (step 2) 98:2 to 30:70 using 1500 mL, and (step 3) 30:70 to 0:100 using 600 mL. Column eluate was collected into 22-mL portions and, on the basis of TLC similarities, recombined into 10 fractions [(1), 1–21, 3.8 mg; (2), 22–26, 353.6 mg; (3), 27–28, 9 mg; (4), 29–35, 91.3 mg; (5), 36–39, 26.8 mg; (6), 40–45, 25 mg; (7), 46–47, 10.8 mg; (8), 48–51, 9.9 mg; (9), 52–58, 6.6 mg; and (10), 59–96, 22.8 mg]. Fractions 4, 7 and 8 were selected for further investigation based on activities in the bioautographic activity against *C. acutatum*, *C. fragariae* and *C. gloeosporioides*. One compound was collected from Fraction 4 and identified as 5-methylmellein.

Compound (–)-5-methylmellein (fraction Ohu19B-F4); high resolution positive ion ESIMS m/z 193.09086 [$\text{M}+\text{H}$]⁺, calculated for $\text{C}_{11}\text{H}_{13}\text{O}_3$, 193.08647; high resolution negative ion ESIMS m/z 191.05360 [$\text{M}-\text{H}$]⁻, calculated for $\text{C}_{11}\text{H}_{11}\text{O}_3$, 191.0708. EIMS (70 eV) m/z [M]⁺ 193 (47), 192 (100), 174 (17.7), 163 (11), 148 (24), 146 (9), 120 (17.8), 91 (17.1). ¹H NMR (400 MHz in CDCl_3) 10.98 s (1H), 7.27 d (1H, $J=8.0$), 6.79 d (1H, $J=8.0$), 4.67 (1H), 2.94 (dd, $J=12.0, 12.0$, 1H), 2.70 (dd, $J=12.0, 12.0$, 1H), 2.14 s (3H), 1.54 d (3H, $J=4.0$). ¹³C NMR (100 MHz in CDCl_3) 170.3 q, 160.4 q, 137.9 s, 137.0 q, 124.9 q, 115.6 s, 108.0 q, 75.4 s, 31.9 d, 20.9 t, 18.1 t. Fraction 7 and 9 were submitted to ¹H NMR analysis and indicated the presence of functional groups associated with fatty acids signals. Subsequently these fractions were investigated by fatty acids analysis.

For the *Alternaria* sp. 3 Ohu13A1 extract 0.765 g was adsorbed to a silica gel chromatography column (40–63 μm , 40 \times 150 mm,

60 Å). Elution of the column was performed using increasing polarity mixtures of chloroform/acetone in a series of four linear steps as follows: (step 1) 100:0 to 99:1 using 600 mL, (step 2) 99:1 to 95:5 using 800 mL, (step 3) 95:5 to 70:30 using 800 mL, (step 4) 70:30 to 0:100 using 800 mL and (step 5) 0:100 using 750 mL (auto extend). Column eluate was collected in 22-mL portions and, on the basis of TLC similarities, recombined into 10 fractions [(1), 1–9, 30.2 mg; (2), 10–34, 444 mg; (3), 35–38, 24.4 mg; (4), 39–43, 156.7 mg; (5), 44–49, 32.1 mg; (6), 50–58, 15.3 mg; (7), 59–62, 38.5 mg; (8), 63–67, 60.6 mg; (9), 68–86, 56.4 mg; and (10), 87–169, 67.5 mg)]. Fractions 4, 7, and 8 were selected for ¹H NMR analysis based on activities in the bioautographic activity against *C. fragariae* and *C. gloeosporioides*. The ¹H NMR analysis indicated the presence of functional groups associated with fatty acid signals. Subsequently these fractions were investigated by fatty acid analysis.

Results

Endophytic fungi identification

A hundred-eight endophytic fungi isolates were obtained, which 46 were recovered from *O. humifusa* collected in the site 1 and 62 in the site 2, representing 17 different taxa identified by molecular methods (Table 1). To confirm the identification of the filamentous fungi, phylogenetic trees of the ITS, TEF1, and/or β -tubulin genes were constructed to illustrate their relationship with GenBank sequences (Figs. 2–4). According to the maximum parsimony analyses generated based on ITS analysis (bootstrap $\geq 94\%$) was evident for the clade containing *B. mediterranea*, *Curvularia protuberata* and *Epicoccum nigrum* (Fig. 2b, d and e). In contrast, the ITS analysis of genus *Alternaria*, *Cladosporium*, *Paraconiothyrium*, *Pestalotiopsis* and *Phoma* did not receive bootstrap support (Fig. 2a, c, and f–h) and their TEF1 and/or β -tubulin sequences (except *Cladosporium*) displayed low sequence similarities when compared with fungal sequences deposited in the GenBank and were identified in genera level. The TEF1 sequences of the *Cladosporium* isolates were analyzed and identified as *Cladosporium cf. asperulatum* and *Cladosporium funiculosum* (Fig. 3b). To *Diaporthe* species, the phylogenetic trees were generated from BA and MP analyses of the combined datasets (ITS and partial sequences of EF 1- α and β -tubulin genes) were highly similar in topology (Fig. 4).

Ecological analyses

A total fungal species richness associated with *O. humifusa* was 17 with $E_{1/D} = 0.86$, suggesting a high diversity of the fungal endophytic community. The distribution of isolates among the 17 species approximated a log-normal pattern ($\text{BIC} = 64.02$), with a few common taxa and many rare taxa. The *O. humifusa* specimens number 3 shelter the dominant endophytic species accounted for 55% of all isolates represented by *Alternaria* sp. 3, *Aureobasidium pullulans* and *Diaporthe* sp. In contrast with these dominant species, about 11.76% (*Paraconiothyrium* sp. and *Pestalotiopsis* sp.) were represented by two isolates, while 47.05% (*Alternaria* sp. 1, *Alternaria* sp. 5, *B. mediterranea*, *Cladosporium cf. asperulatum*, *Cryptococcus flavescens*, *E. nigrum* and *Phoma* sp.) were unique, representing singlettons. Additionally, the species richness obtained indicates that the sample effort was enough to recover the diversity pattern obtained (Fig. 5). As an example that supports this assertion, is the number of added species or extrapolated richness (S_p) and their standard errors encountered in Bootstrap ($S_p = 20.10 \pm 1.35$) and Jackknife 1 ($S_p = 24.76 \pm 2.75$).

Comparisons between endophytic assemblages from sites 1 and 2 showed heterogeneity and significant difference in species

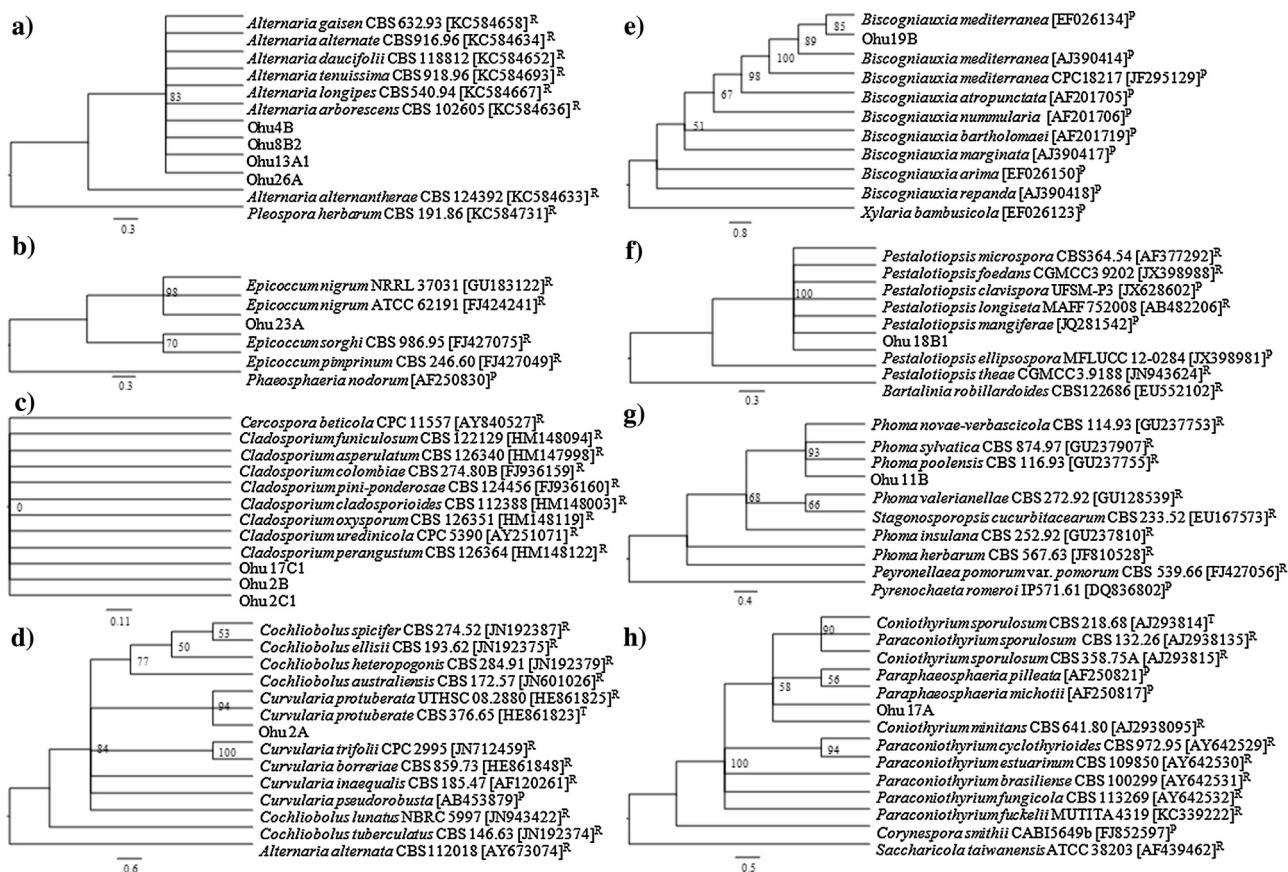


Fig. 2. Maximum parsimony bootstrap ITS trees of fungal endophytes isolated from the *Opuntia humifusa*, Missouri, USA, *Alternaria* (a), *Epicoccum* (b), *Cladosporium* (c), *Curvularia* (d), *Biscogniauxia* (e), *Pestalotiopsis* (f), *Phoma* (g) and *Paraconiothyrium* (h).

composition (ANOSIM $R=0.46$, $P<0.001$) with $B(W)=0.48$. Most endophytic richness occurred between plants ($D_{ST}=0.874$). The site 1 not only is the most diverse but also has the most unique species composition, and the site 2 lowest contribution to the gamma diversity. In addition, six species were common to both sites; eight species (*C. protuberata*, *C. funiculosum*, *Alternaria* sp. 1, *Alternaria* sp.

5, *C. flavescens* and *Phoma* sp.) were exclusive to site 1; and three species (*Alternaria* sp. 4, *B. mediterranea*, and *E. nigrum*) exclusive to site 2.

The biological activities and chemical analyses

Six extracts (5.5%) of fungal endophytes showed antifungal properties with zones of inhibition ranging from 4.5 to 11.2 mm in the bioautography assay (Table 2). ^1H NMR analyses of the bioactive extracts of *Alternaria* sp. 5 Ohu 8B2, *Alternaria* sp. 3 Ohu 30A, *C. funiculosum* Ohu 17C1 and *Paraconiothyrium* sp. Ohu 17A indicated the presence of functional groups associated with unsaturated fatty-acid olefinic protons and fatty acid methylene and methyl protons. GC-FID analysis of these extracts confirmed the presence of a mixture of fatty acids (Table 3).

The ^1H NMR analyses of the crude extracts obtained from *B. mediterranea* Ohu 19B and *Alternaria* sp. Ohu 13A1 showed the presence of aromatic compounds, which were therefore purified using bioassay-guided fractionation. However, ^1H NMR analyses of the bioactive fractions 4, 7, and 8 from *Alternaria* sp. Ohu 13A1 showed the presence of a mixture of fatty acids inside of aromatic compounds. These results were confirmed by GC-FID analysis (Table 3). One aromatic compound was collected from Fraction 4 from *B. mediterranea* Ohu 19B and identified as 5-methylmellein. ^1H and ^{13}C NMR data for Ohu19B-F4 indicated complete agreement with that previously reported (Carpenter et al. 1980) providing structural confirmation of fraction Ohu19B-F4 as (−)-5-methylmellein. The other bioactive fractions 4 and 8 from *B. mediterranea* Ohu 19B showed the presence of a mixture of fatty acids by ^1H NMR analyses and these results were confirmed by GC-FID analysis (Table 3).

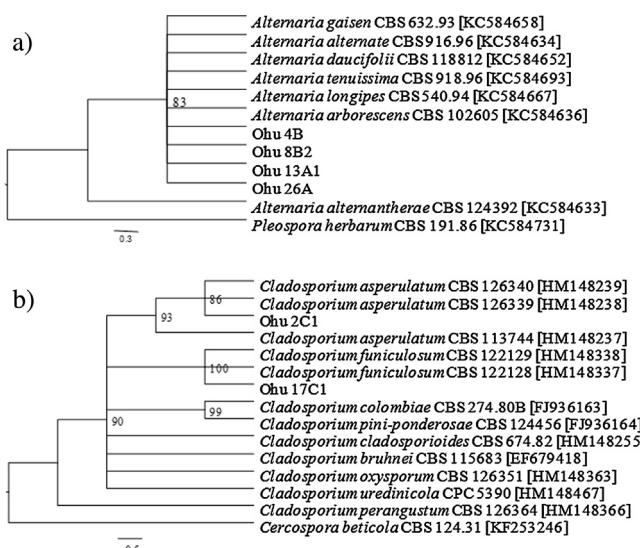


Fig. 3. Maximum parsimony bootstrap TEF1 trees of fungal endophytes isolated from the *Opuntia humifusa*, Missouri, USA, *Alternaria* (a), *Cladosporium* (b).

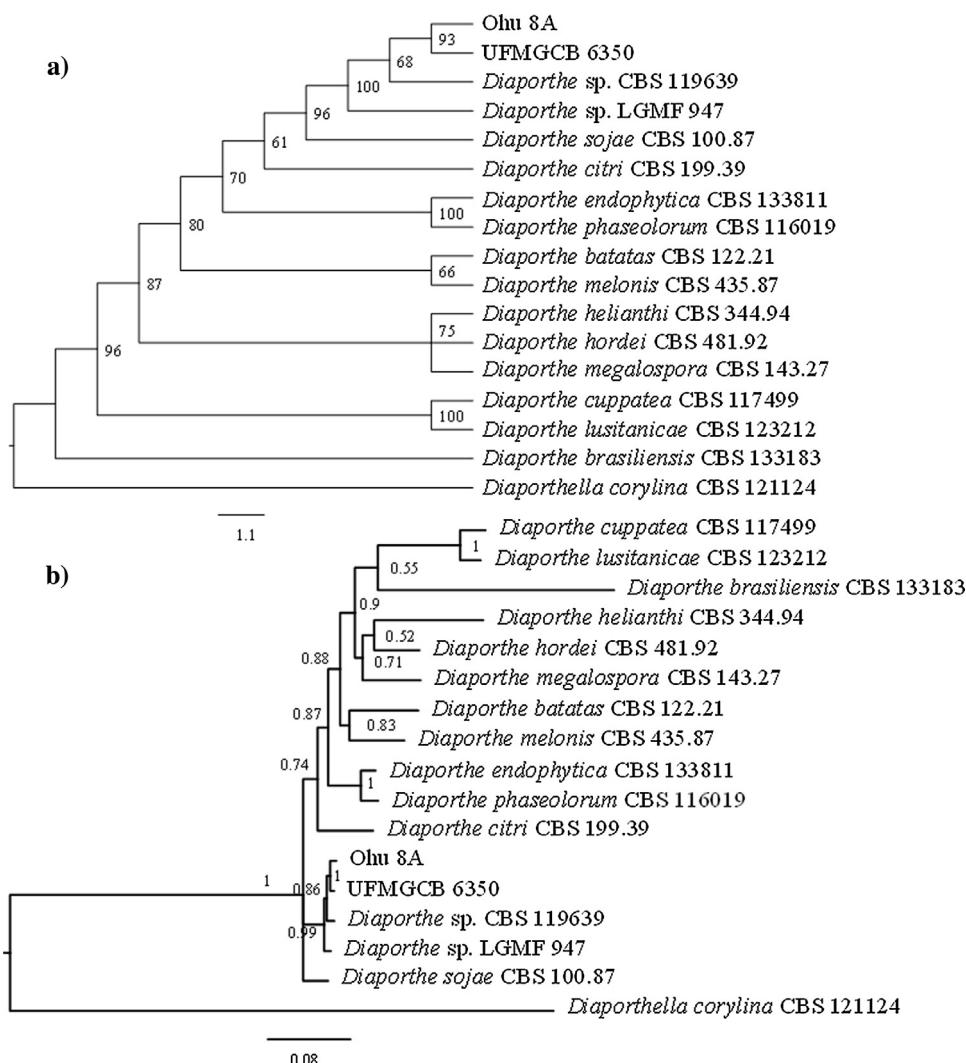


Fig. 4. Maximum parsimony (a) and the Bayesian analysis (b) trees of the combined 3-gene sequence alignment (ITS, TEF1 and β -tubulin) for *Diaporthe* species. The tree was rooted to *Diaporthella* corylina.

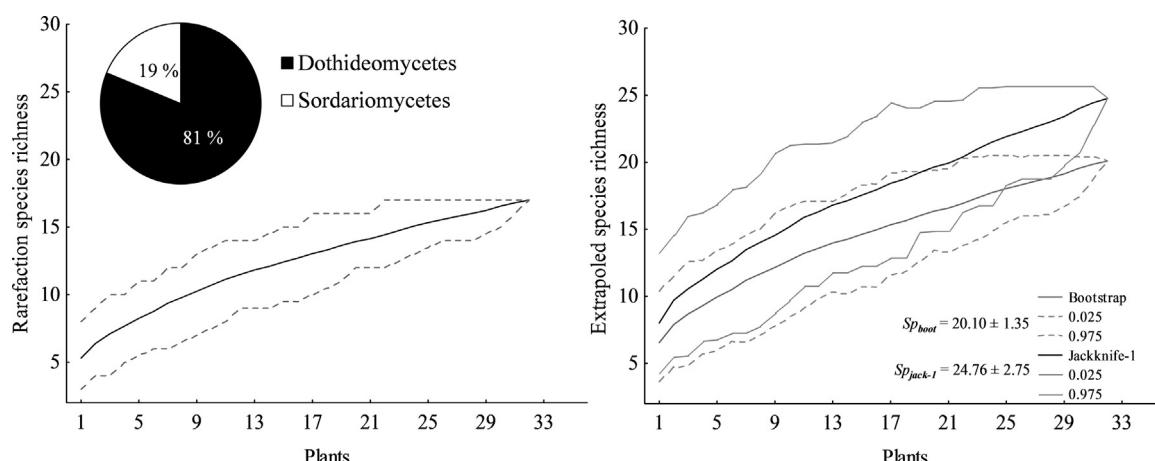


Fig. 5. Curve of species accumulation using the individual-based rarefaction and inset pie charts indicate the taxonomic distribution of endophytes among classes of Ascomycota (left) and extrapolated species richness (S_p) with standard errors in a species pool based on two estimators: first order jackknife and bootstrap (right). In all analysis 999 randomizations was performed.

Table 2

Antifungal activities of extracts from endophytic fungi isolated from *Opuntia humifusa* using direct bioautography against three *Colletotrichum* target species.

Ohu	Taxon	Mean fungal growth inhibition (mm) \pm SE [†]		
		<i>C. fragariae</i>	<i>C. gloeosporioides</i>	<i>C. acutatum</i>
8B2	<i>Alternaria</i> sp. 1	6 \pm 2.82	6.5 \pm 3.53	4.5 \pm 0.7
13A1	<i>Alternaria</i> sp. 3	8 \pm 0	9 \pm 0	8 \pm 0
30A	<i>Alternaria</i> sp. 3	4.5 \pm 0.7	4.5 \pm 0.7	11 \pm 9.8
19B	<i>Biscogniauxia mediterranea</i>	10 \pm 0	Diffuse zone	9 \pm 1.4
17C1	<i>Cladosporium funiculosem</i>	Diffuse zone	Diffuse zone	4.5 \pm 0.7
17A	<i>Paraconiothyrium</i> sp.	5.5 \pm 0.7	5.5 \pm 0.7	11.25 \pm 7.4
Controls	Benomyl	0 \pm 0	0 \pm 0	0 \pm 0
	Cyprodinil	29.75 \pm 0.35	25.25 \pm 1.06	30.5 \pm 4.9
	Azoxystrobin	22 \pm 2.82	30 \pm 0	21 \pm 4.24
	Captan	12.25 \pm 1.76	12.75 \pm 0.35	13.5 \pm 2.12

[†] Mean inhibitory clear zones and standard errors were used to determine the level of antifungal activity against each fungal species.

[§] Spots of the technical-grade fungicides benomyl (at 1.16 g), cyprodinil (at 0.9 g), azoxystrobin (at 1.61 g), and captan (at 1.2 g) diluted in ethanol were used as standard controls.

The 5-methylmellein was evaluated for antifungal activity against seven plant pathogens using an *in vitro* microdilution broth assay. The most sensitive species were those in the genus *Phomopsis*, with *P. obscurans* being most susceptible (63.5% growth inhibition). The best growth inhibition (20.1%) to *F. oxysporum* was 300 μ M at 48 h. Lower doses (75 and 150 μ M) of this compound caused stimulation of *B. cinera* and *C. fragariae*, while all doses caused stimulation of *C. acutatum* and *C. gloeosporioides* (Figs. 6 and 7).

Discussion

The endophytic colonization of *O. humifusa* was similar with the results found in other studies with *Cactaceae* obtained by Fisher et al. (1994), Suryanarayanan et al. (2005), Bezerra et al. (2012), and Bezerra et al. (2013). The most of the fungal genera obtained as endophytes of *O. humifusa* were described as endophytes and the genera *Alternaria*, *Aureobasidium*, *Cladosporium*, *Cochliobolus/Curvularia*, *Pestalotiopsis*, *Phoma* and *Xylaria*

were found in association cacti. However, among the fungi identified as endophyte of *O. humifusa*, the genera *Biscogniauxia*, *Cryptococcus*, *Diaporthe* and *Paraconiothyrium* and the species *B. mediterranea*, *Cladosporium cf. asperulatum*, and *C. protuberata* were reported for the first time as endophytic of cacti.

Basidiomycota are rarely isolated (Chlewicki 2009) as endophytes and associated with cacti are known only eight species (*Rhodotorula foliorum*, *R. minuta*, *R. mucilaginosa*, *R. pilatii*, *R. sonckii*, *Sporobolomyces salmonicolor*, *Sterigmatomyces elviae* and *Tritirachium dependens*), which were recovered from cladodes of *Cereus jamacaru* (Bezerra et al. 2013). In this study, we recovered *C. flavescent* (*Tremellomycetes, Basidiomycota*) that represent the first report as endophytes associated with *Cactaceae*. *Cryptococcus* species are described as endophytes, including *C. flavescent* recovered from *Citrus sinensis* (Gai et al. 2009; Herrera et al. 2010).

The taxa *A. pullulans*, *Alternaria* sp. 3 and *Diaporthe* sp. were the species most frequently found in *O. humifusa*. These results were similar to those found by Suryanarayanan et al. (2005), which

Table 3

Fatty acids analysis and quantification produced by endophytic fungi (extracts and fractions).

Fatty acids	Extracts				Ohu 13A1 fractions			Ohu 19B fractions	
	Ohu 8B2	Ohu 30A	Ohu 17C1	Ohu 17A	F7	F8	F9	F7	F9
Caprylic (8:0)	0.85	0.32	–	–	–	–	–	–	–
Capric (10:0)	–	–	2.22	–	–	–	–	–	–
Undecanoic (11:0)	–	–	2.89	–	–	–	–	–	–
Lauric (12:0)	–	–	36.68	–	–	0.11	0.46	–	–
Myristic (14:0)	0.64	0.52	–	0.82	0.19	0.68	1.39	0.16	0.17
Pentadecanoic (15:0)	1.29	1.57	–	0.59	–	–	–	–	–
Palmitic (16:0)	47.83	32.74	42.75	45.99	24.9	36.3	43.84	8.61	2.80
Palmitoleic (16:1)	0.96	1.14	–	2.47	2.27	2.71	1.97	1.43	2.18
Heptadecanoic (17:0)	–	–	–	–	0.51	0.1	0.66	–	1.58
cis-10-Heptadecenoic (17:1)	–	–	–	–	0.21	0.18	–	–	–
Stearic (18:0)	16.51	11.14	15.43	4.04	5.37	5.22	5.05	0.07	–
Oleic (18:1n9c)	26.01	20.36	–	17.19	20.92	17.53	9.7	4.28	0.79
(LA) Linoleic (18:2n6c)	4.48	30.96	–	27.27	42.88	34.66	17.04	81.86	75.79
Pinolenic (18:3n6)	0.58	0.37	–	–	–	–	–	–	–
(ALA) alpha-Linolenic (18:3n3)	–	–	–	–	2.71	2.32	1.21	1.98	3.05
Nonadecanoic (19:0)	–	0.4	–	1.38	–	–	–	–	0.53
Arachidic (20:0)	0.8	0.41	–	0.21	–	–	5.71	0.91	2.12
cis-11-Eicosenoic (20:1)	–	–	–	–	0.13	–	–	–	–
Sciadonic (20:3n6)	–	–	–	–	–	–	–	–	8.42
Arachidonic (20:4n6)	–	–	–	–	–	–	1.1	–	–
cis Eicosapentaenoic (20:5n3)	–	–	–	–	–	–	1.63	–	–
cis-13-docosadienoic (22:2)	–	–	–	–	–	–	3.79	–	–
Docosapentaenoic (22:5n3)	–	–	–	–	–	–	2.82	–	–
Lignoceric (24:0)	–	–	–	–	–	–	3.57	0.49	2.54

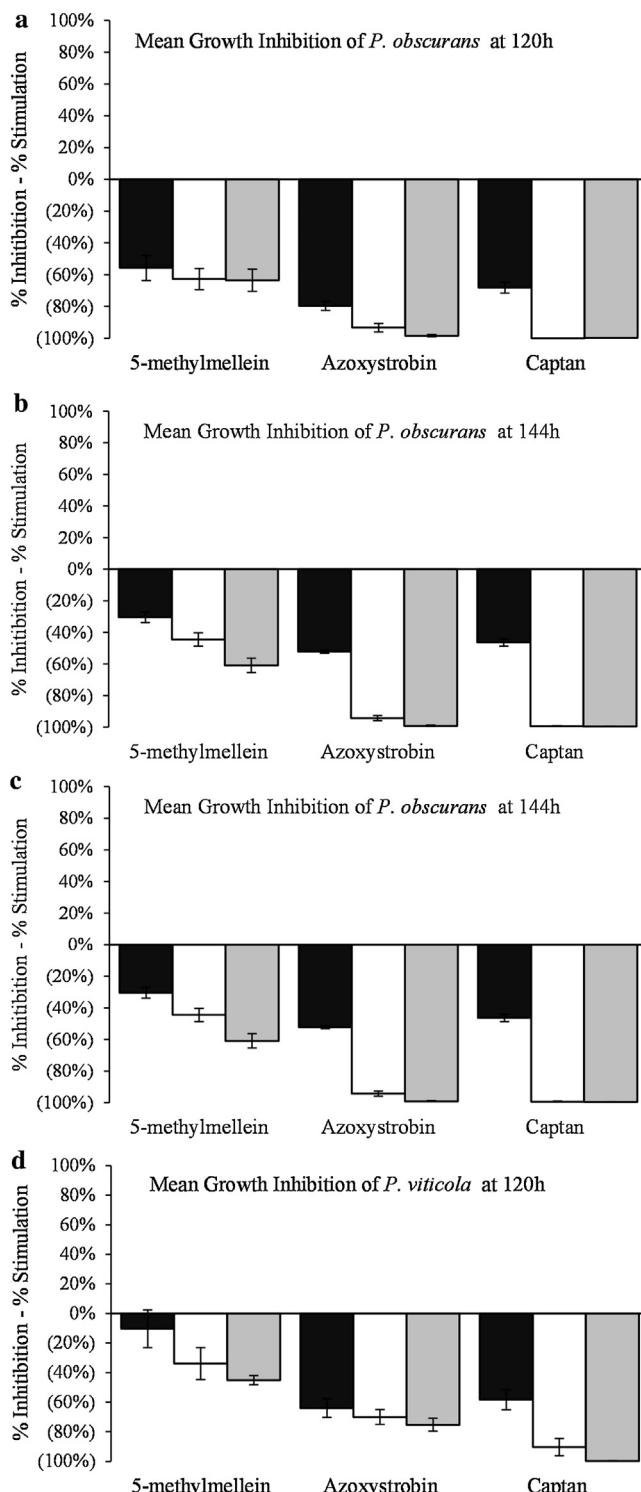


Fig. 6. Mean fungal growth inhibition (%) of *Phomopsis obscurans* (a, b) and *Phomopsis viticola* (c, d) after exposure to compounds 5-methylmellein at the concentrations of 75 (black bar), 150 (white bar) and 300 (gray bar) μM using a dose-response format at 48 and 72 h, respectively. Fungicide standards: captan and azoxystrobin at the concentrations of 0.3 (black bar), 3.0 (white bar) and 30 (gray bar) μM. Means from percent growth inhibition/stimulation were pooled from two experiments replicated in time.

obtained *Alternaria* sp. and *A. pullulans* as the species most frequently several cacti from Arizona (USA). The *Diaphorte* sp. Ohu 8A isolates recovered from *O. humifusa* formed the monophyletic clade genetically closed to *Diaporthe* sp. CBS 119639 and *Diaporthe* sp. LGMF 947. *Diaporthe* sp. CBS 119639 was isolated from an abscess of a male patient in Germany and *Diaporthe* sp. LGMF 947 obtained from soybean seeds in Brazil (Gomes et al. 2013). The phylogenetic studies suggest that these strains represent a new species, including *Diaphorte* sp. Ohu 8A.

Different bioactive extracts of the endophytic fungi associated with *O. humifusa* showed the presence of fatty acids, which are known to possess antifungal activity (Kabara et al. 1972; Rosa et al. 2013). The lauric acid (saturated fatty acid), identified in this work as the majority fatty acid to crude extract *C. funiculosum* Ohu 17C1, which display antifungal activity against different plant pathogens fungi (Kabara et al. 1972; Murzyn et al. 2010).

The palmitic acid (saturated fatty acid), identified in this work as the majority fatty acid to crude extracts *Alternaria* sp. 1 Ohu 8B2, *Alternaria* sp. 3 Ohu 30A, *C. funiculosum* Ohu 17C1 and *Paraconiothyrium* sp. Ohu 17A, and to fractions 8 and 9 from crude extract *Alternaria* sp. 3 Ohu 13A1, show antifungal activity described in against *Alternaria solani*, *Aspergillus niger*, *A. terreus*, *Cucumerinum lagenarium*, *Emericella nidulans* and *Fusarium oxysporum* (Liu et al. 2008; Altieri et al. 2007, 2009). The linoleic acid (unsaturated fatty acid) was majority in the fractions 7 and 9 from *B. mediterranea* Ohu 19B, in the fractions 7 and 8 from *Alternaria* sp. 3 Ohu 13A1, as well as to crude extract *Alternaria* sp. 3 Ohu 30A. Linoleic acid is well-known for has antifungal activity against several phytopathogenic fungi, such as *A. solani*, *Colletotrichum lagenarium*, and *F. oxysporum* (Liu et al. 2008). The oleic acid (also unsaturated fatty acid) was detected in the crude extracts *Alternaria* sp. 1 Ohu 8B2, *Alternaria* sp. 3 Ohu 30A, and *Paraconiothyrium* sp. Ohu 17A, as well as in the fractions from *Alternaria* sp. 3 Ohu 13A1. The oleic acid was reported as antifungal against the phytopathogenic *Moniliophthora perniciosa* and *Pythium ultimum* (Walters et al. 2004). Similar to our results, Rosa et al. (2013) identified a mixture of several saturated and unsaturated fatty acids in the fractions from extract of endophytic fungus *Coniochaeta lignaria* and the host plant *Smallanthus sonchifolius*, which showed antifungal activities against the phytopathogenic *C. acutatum*, *C. fragariae* and *C. gloeosporioides*.

The chemical bioassay-guided purification of a crude extract of *B. mediterranea* Ohu 19B resulted in the identification of (–)-5-methylmellein, a dihydroisocoumarin, which was described as metabolites of different fungi, including *B. mediterranea* (Anderson et al. 1983; Ahmed et al. 2011). *B. mediterranea*, the causal agent of the well-known charcoal disease, is widely spread in mediterranean region affecting mainly several *Quercus* species, but also was reported as endophyte (Santos 2003). According to Nugent et al. (2005), there is evidence that these species occur in healthy living trees as endophytes and then become invasive under water stress conditions. The (–)-5-methylmellein has been described as antibacterial and antifungal compounds against different microorganisms (Krohn et al. 1997; Kokubun et al. 2003). In our study the microdilution broth assay result demonstrated that (–)-5-methylmellein showed antifungal activity against *P. viticola* and *P. obscurans* (inhibition 50–63%, respectively) and weak antifungal activity (inhibition to 20%) against *B. cinerea*, *C. fragariae* and *F. oxysporum* at 300 μM. These results suggested that (–)-5-methylmellein and majority fatty acids found in fractions 7 and 9 can act in synergy and be responsible for the bio-activity initially observed in the crude extract. Our results suggest that native medicinal cacti of United States can live symbiotically with rich and diverse endophytic communities and may be sources of bioactive molecules, including those able to inhibit or control plant pathogens diseases.

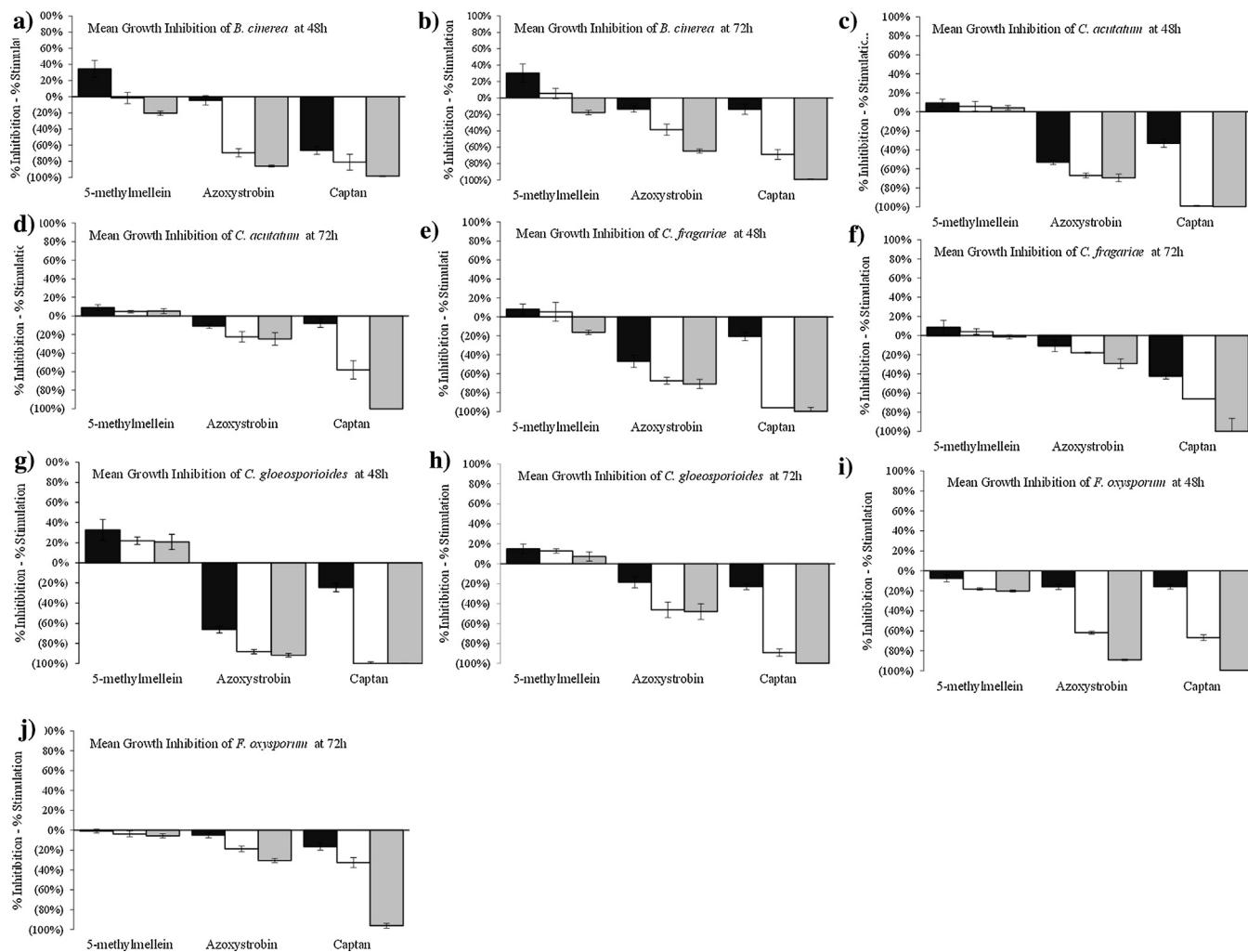


Fig. 7. Mean fungal growth inhibition/stimulation (%) of *Botrytis cinerea* (a, b), *Colletotrichum acutatum* (c, d), *Colletotrichum fragariae* (e, f), *Colletotrichum gloeosporioides* (g, h) and *Fusarium oxysporum* (i, j) after exposure to compounds 5-methylmellein at the concentrations of 75 (black bar), 150 (white bar) and 300 (gray bar) μ M using a dose-response format at 48 and 72 h, respectively. Fungicide standards: captan and azoxystrobin at the concentrations of 0.3 (black bar), 3.0 (white bar) and 30 (gray bar) μ M. Means from percent growth inhibition/stimulation were pooled from two experiments replicated in time.

Acknowledgements

Visiting Graduate Scholar Alice F. S. Hughes was financially supported by the Brazilian CAPES Foundation (Capes PSDE 1283/12-0), CAPES (23038.003478/2013-92) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 141684/2010-0). The National Center for Natural Products Research, University of Mississippi is partially supported through a cooperative scientific agreement 58-6408-2-0009 from the USDA, Agricultural Research Service. The authors thank Mrs. Ramona K. Pace, Ms. Linda J. Robertson, Mrs. Amber Reichley, Mr. Solomon Green III and Mrs. Marilyn Ruscoe for technical support. We also thank Dr. Frederic M. Hughes for the valuable help on the phylogenetic and statistical analysis.

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